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Pharmacophore Modeling Strategies for the Development of Novel Nonsteroidal Inhibitors of Aromatase (CYP19)

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Background and Significance

Breast cancer, the most frequently diagnosed cancer affecting women [1], ranks second to lung cancer in total tumor-related deaths. Currently, one in eightAmerican women will develop invasive breast cancer during her life. Approximately two-thirds of breast cancer tumors are hormone-dependent and require growth factors like estrogen to develop [2]. Estrogens are synthesized in the human body from cholesterol through a multi-step reaction catalyzed by the enzyme aromatase.

Once synthesized in the body, estrogens bind to estrogen receptors [2]. These ligand-receptor complexes then homodimerize and eventually lead to the transcription of growth-stimulating proteins [2,3]. These proteins then cause the cell to grow and divide and can even influence neighboring cells to do the same [2], thus causing a tumor to form. Endogenous biosynthesis of estrogen is catalyzed by aromatase, a member of the cytochrome P450 (CYP450) superfamily of monooxygenase enzymes. Although members of this family have several features in common, aromatase in particular is the only member with less than 20% sequence identity when compared with others in the superfamily [4].

Aromatase, also known as estrogen synthase or CYP19 [4], is the only vertebrate enzyme known [5] to catalyze the biosynthetic conversion of 19-carbon androgens – like testosterone and androstenedione – into 18-carbon estrogens [1,2,5,6-9] like estradiol and estrone, respectively [4,6,9]. Anchored to the plasma membrane of the endoplasmic reticulum [4,5,8,10], aromatase performs three oxidative steps [2,4,5,9], the last of which being the rate-limiting aromatization of the steroidal A-ring [4-6,8,9] that is specific only to this enzyme [5].

Several classes of aromatase inhibitors (AIs) have been developed in the past two decades, despite lack of experimental structural data. On the basis of their inhibition mechanism and chemical origin, these molecules are divided into two classes: steroidal (type I) and non-

steroidal (type II) [2,1]. Steroidal AIs are derivatives or analogues of the preferred androgenic substrates and inhibit aromatase irreversibly. They can be further divided into competitive inhibitors and mechanism-based inhibitors. Competitive inhibitors bind non-covalently to aromatase in a manner similar to that of the natural substrate and block it from being enzymatically modified by aromatase. Mechanism-based inhibitors, also known as suicide inhibitors, bind to the enzyme, are converted into a reactive intermediate that covalently binds the enzyme and permanently inactivates it, often destabilizing it and increasing its rate of degradation by the intracellular proteosome [1,9].

Nonsteroidal inhibitors, on the other hand, bind entirely noncovalently to the enzyme and are flavonoids-derivatives containing a heteroatom that coordinates with the iron atom of the heme group [2,1] to block the active site and reversibly inhibit the enzyme. These types of AIs are further divided into categories based on the order in which they were discovered or synthesized: first-, second-, and third-generation AIs. Currently, the third-generation of triazole-derived AIs are approved as front-line therapy for early or even advanced cases of breast cancer in postmenopausal women [8,9].

However, for both steroidal and non-steroidal AIs, important side effects – ranging from mild to severe, short-term to long-term – have been suggested or reported. For example, steroidal AIs often give way to androgenic side effects where other related systems are disturbed due to the AI's lack of specificity [10]. Also, prolonged estrogen deprivation can lead to bone loss, osteoporosis, reproductive problems, or even other types of cancers [9]. Therefore, more selective and less toxic CYP19 inhibitors are needed, especially since mutations in intratumoral aromatase can cause changes in its stability, efficiency, or sensitivity to different classes of AIs and can vary from patient to patient [1].

Until recently, the microsomal nature of aromatase has made it difficult to crystallize. Homology models of human aromatase were created based on other, slightly similar P450 enzymes like rabbit progesterone 21-hydroxylase CYP2C5, the first mammalian CYP450 to be crystallized and sequenced [4]. However, due to low sequence similarity between human aromatase and the other members of the cytochrome P450 superfamily [7], homology models have not been able to shed much light upon the structure of the human aromatase.

The recent determination of the crystal structure of aromatase complexed with androstenedione [5] defines a few striking structural characteristics such as the importance of a diverse set of residues and the various complicated functions of a few key residues [4,5,7,9]. Of particular importance are residues like Arg115 [5,9], Ile133 [5,7,9], Glu245 [7], Glu302 [7], Ala306 [5], Asp309 [5,7,9], Thr310 [5,7], Met374 [5,9], and Asp476 [7].

Many residues – whether comprising the active site or situated quite far from it – play essential roles stabilizing the enzyme, positioning the substrate, or catalyzing the multi-step reaction. The catalytic cleft of the enzyme is open to the outside via an access channel devoid of solvent, although networks of water molecules hydrogen-bonded to important residues are predicted to aid in enzyme catalysis [5].

Androgens, the preferred substrates of aromatase, are believed to enter the enzyme's catalytic cleft and active site through an access channel open to the outside environment of the enzyme and most likely devoid of solvent [5]. The catalytic cleft encompasses a volume of 400Å^3 or less, while the active sites of other related aromatase enzymes are surrounded by a volume of about 530Å^3 [5]. Due in part to this relatively smaller volume and also to the diverse set of amino acid interactions defining the enzyme, the catalytic cleft of aromatase is very specific in its complementarity to its steroidal substrate [5].

Additionally, of particular importance is the heme group, comprised of a porphyrin ring and an iron atom as part of an oxyferryl moiety, Fe(IV)=O [5]. Under nonsteroidal inhibition, the heme moiety is specifically targeted, binding heterocycle atoms of the AI [1,2,6,8,11,12] and making it its sixth ligand [6]. Steroidal inhibition also affects the heme group, although not quite as directly. The heme moiety and a few of the important residues are shown in ***Figure 1***.

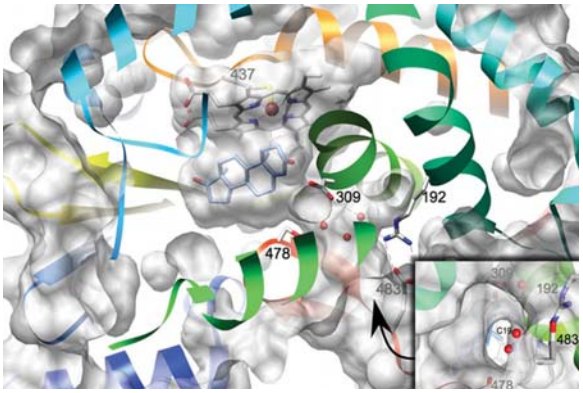


Figure 1: Active site of CYP19 showing heme moiety, Arg192, Asp309, Cys437, Ser478, and Glu483 [5].

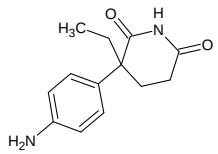
Therefore, the recent determination of the crystal structure of aromatase, which reveals the molecular basis for the enzyme's androgenic specificity and unique catalytic mechanism [5], provides an excellent and timely opportunity to design the next generation of aromatase inhibitors.

Research Design and Methods

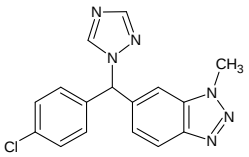
A 3-dimensional (3D) pharmacophore model of the CYP19 binding pocket will be created with Ligand Scout [13] using the x-ray structure deposited in the Protein Data Bank (PDB) [pdb code: 3EQM]. The model will be based on interactions that define aromatase inhibition, such as hydrophobic interactions, hydrogen bonding, and electrostatic interactions [13,14]. Features identified by the LigandScout software are those that take into consideration chemical functionality but not strict structural topology or definite functional groups. As a result, completely new potential pharmacons can be identified through database screening. The generated pharmacophore model will be validated against a test set consisting of strong and weak AIs.

In addition to a structure-based pharmacophore developed with LigandScout, a ligand-based pharmacophore developed with Molecular Operating Environment (MOE) will also be generated. The Pharmacophore Elucidation algorithm of MOE [15] will be used to generate this model with conformations of the set of training inhibitors shown in ***Figures 2a*** and ***2b***. This set of AIs includes structurally diverse, strongly inhibitory molecules in addition to weak or inactive aromatase inhibitors. Favorable pharmacophore features will be derived from the active inhibitors and unfavorable features will be derived from the inactive molecules.

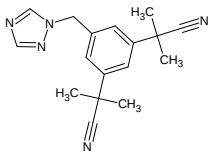
Training Set: Active Aromatase Inhibitors



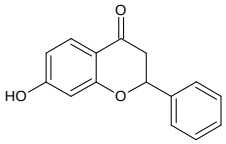
Fadrozole [16] 5nM



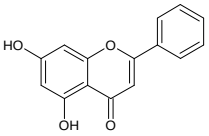
Vorozole [17] 1.38 nM



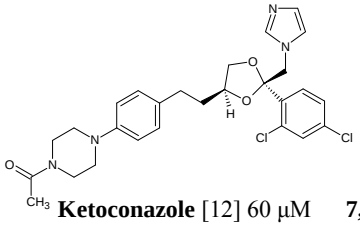
Anastrozole [2] 15 nM



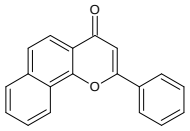
7-Hydroxyflavone [18] 30.5 μM



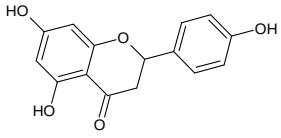
Chrysin [18,19] 1 μM



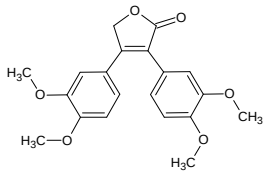
Ketoconazole [12] 60 μM



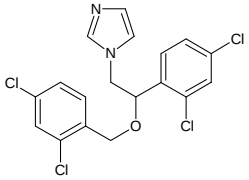
7,8-Benzoflavone [20] 0.07 μM



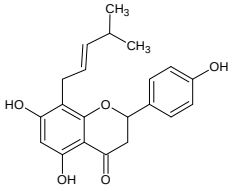
Naringenin [19] 2.9 μM



TM-8 [19] 1.0 μM



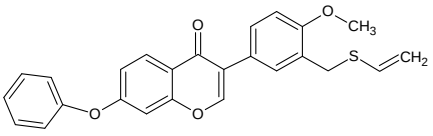
Miconazole [12] IC50 = 0.6 μM



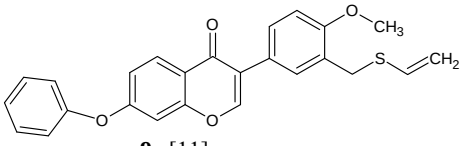
4',5,7-OH-8-prenylnaringenin [19] 0.2 μM

Figure 2a: Training set of active aromatase inhibitors with corresponding IC_{50} values in human placental microsomes.

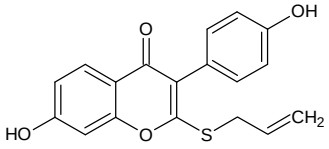
Training Set: Inactive or Poor Aromatase Inhibitors



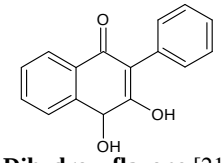
9a [11]



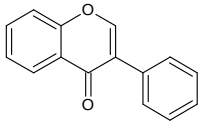
9c [11]



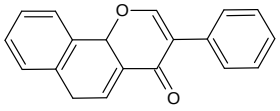
12b [11]



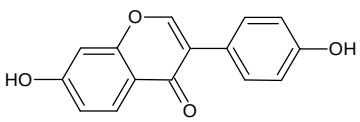
3',4'-Dihydroxyflavone [21]



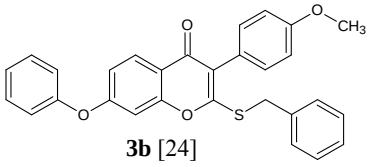
Isoflavone [21]



5,6-Benzoflavone [23]



Daidzein [22]



3b [24]

Figure 2b: Training set of inactive aromatase inhibitors.

After this model is generated, it will be validated using the test set of aromatase inhibitors shown below in ***Figure 3***.

Test Set: Active Aromatase Inhibitors

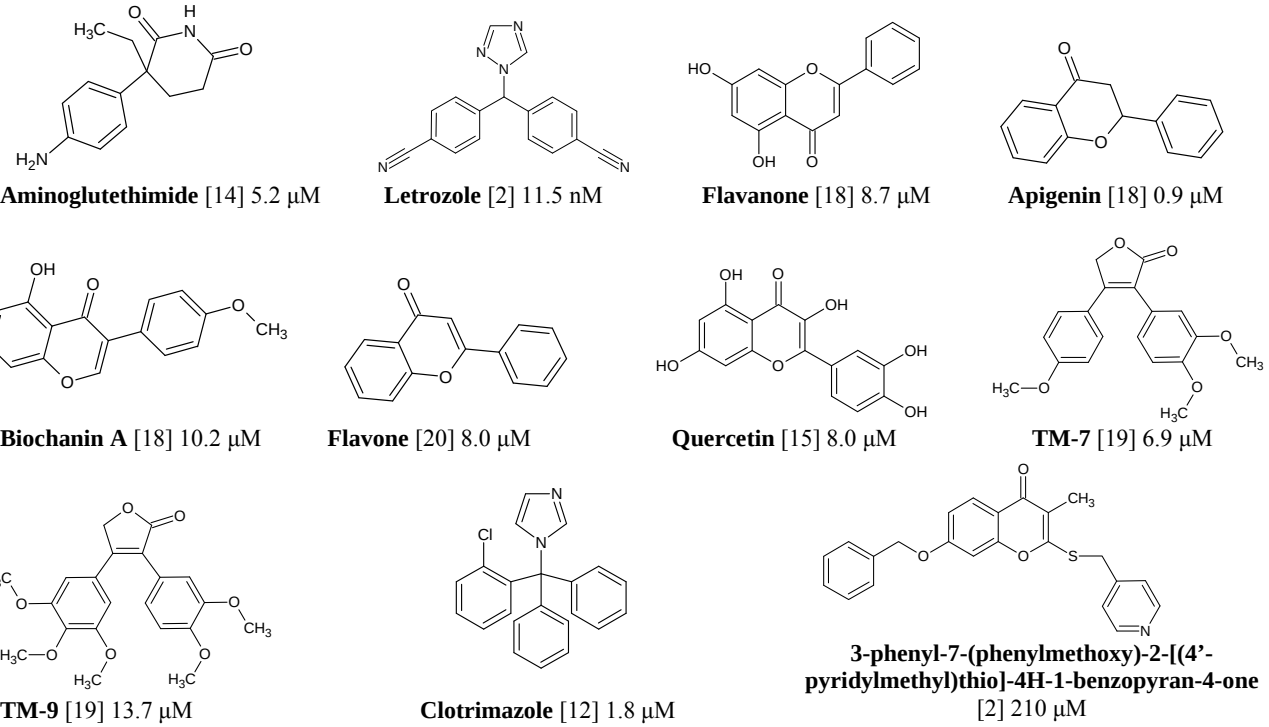


Figure 3: Test set of active aromatase inhibitors with corresponding IC_{50} values in human placental microsomes.

Following its validation, the ligand-based pharmacophore model will then be merged with the structure-based model created through Ligand Scout. The combination of these two approaches will highlight the importance of chemical features through the ligand-based pharmacophore as well as the importance of enzyme-ligand interactions through the structure-based model. By merging these two pharmacophore models, databases of commercially available small molecules – like the ZINC database [25] – can be screened for new potential aromatase inhibitors. The hits will be prioritized using ADME and toxicity screening. The top 20 compounds from this prioritized list will then be tested experimentally using standard CYP19 *in vitro* assays.

A schematic diagram depicting this strategy, divided into Phases I and II, is shown in ***Figure 4***:

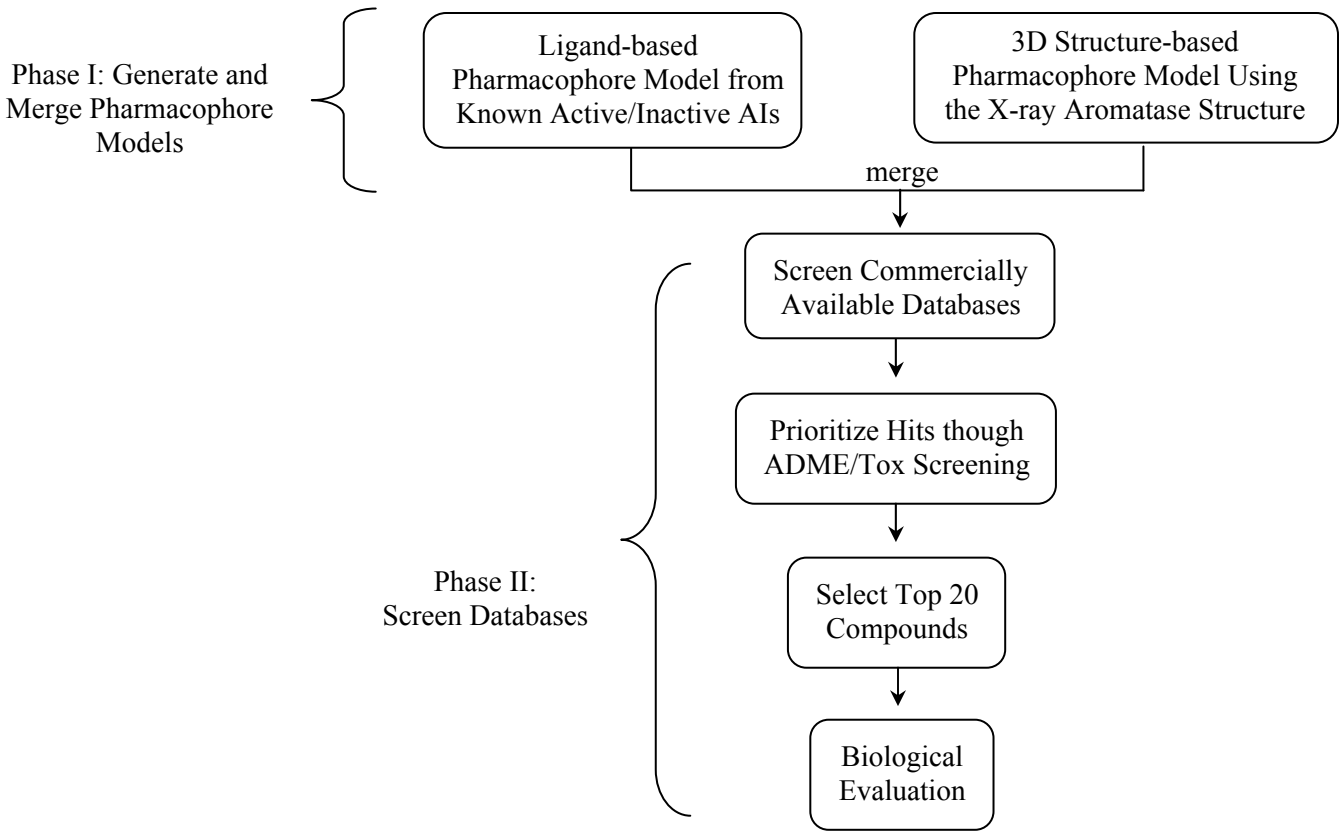


Figure 4: A schematic diagram depicting the strategy to be employed.

Expected Outcome

By combining a ligand-based model and a structure-based model into one pharmacophore, the outlined approach not only highlights the importance of chemical features of known aromatase inhibitors but also of the enzyme-ligand interactions necessary for effective inhibition. As a result, the identification of more potent inhibitors is expected in the long run, although top hits from database screenings will need to undergo analysis for toxicity and *in vivo* efficacy. The proposed study will help identify the next generation of potential aromatase inhibitors for breast cancer treatment.

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